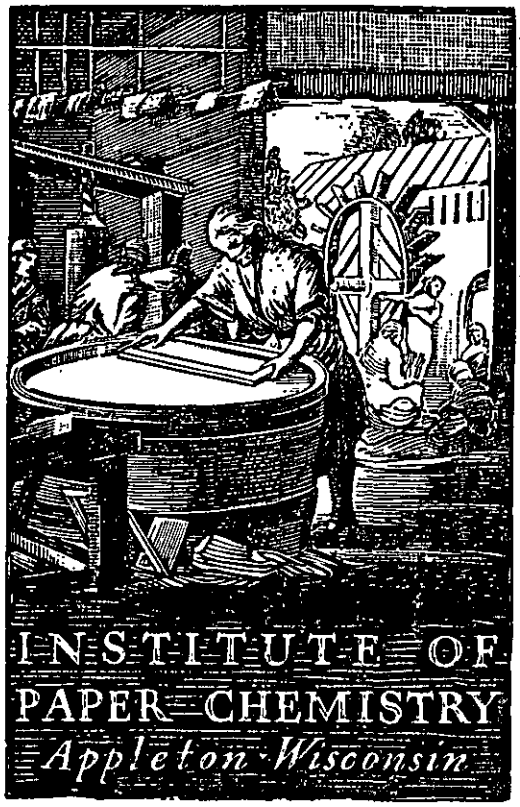

ASPEN TREES FROM TISSUE CULTURE

Project 2351

Report Nine

A Progress Report

to

PIONEERING RESEARCH COMMITTEE

October 10, 1969

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

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SUMMARY

A major breakthrough was accomplished with the first reproduction of a tree species from tissue culture. Four triploid quaking aspen trees (Populus tremuloides Michx.), now several feet tall, survived from seven complete plants produced on firm white callus tissue. Unrooted shoots were also cut from callus and rooted in medium or soil, and two also grew into trees. Additional studies were run on the effects of amino acid and nucleotide supplements on tissue growth and shoot production. Amino acids stimulated tissue growth after four weeks in culture, but the best shoot production occurred on inocula cut from stock tissue grown for four weeks or less from subculture. Nucleotides stimulated the growth of stock tissue less than four weeks old, specifically guanylic acid alone, and to a lesser degree adenylic acid. The highest percentage of inocula with shoots also occurred on tissue transferred from medium supplemented with guanylic acid to medium without auxin but containing 0.15 mg./l. 6-benzylamino-purine. The possible interactions of amino acids and nucleotides on gene regulation of cell enlargement as opposed to cell differentiation were discussed, pointing out the suitability of the aspen cultures for basic studies in molecular botany.

INTRODUCTION

Last year in Report Eight, we described the first known complete plants of a tree species grown on tissue cultures of triploid quaking aspen (1). Since then, Aneja and Atal (2) in India reported plantlets from tissue cultures of eucalyptus, and a student in California recovered plantlets from cultures of ginkgo (unpublished). The "plantlets" reported by Mathes (3), Winton (4) and Wolter (5) were really pieces of undifferentiated callus tissue with roots and shoots that were not attached to each other. Wolter and Mathes both cut shoots from the callus and obtained roots from their base in a fresh medium, as we also did last year, but these were rooted cuttings and not complete plants produced on callus.

The major breakthrough during the past year, here at the Institute, was the successful elongation of the shoot and root of some of our complete plants, and the first reproduction of a tree from tissue culture. Neither Wolter nor Mathes have accomplished this, and at a recent meeting of world botanists in Seattle, Washington no one challenged the claim, that as far as could be determined this was the first successful reproduction of a tree from tissue culture.

In May 1969, the four trees, surviving from seven complete plants, were displayed at the Executives' Conference at The Institute of Paper Chemistry. This was preceded by one month, by a press conference and release to public news media of a report and photographs of the trees. Mr. Arnold Grummer, Director of Special Publications, conducted a responsible and tightly-controlled publicity program to both dramatize the trees and to extend an awareness to the public of the function and scope of The Institute of Paper Chemistry. The response was much greater than anticipated.

The news release or parts thereof, or original stories written from personal communications, have so far appeared in over 80 newspapers and a dozen scientific, technical or popular journals, plus taped interviews on radio ABC nationally, and television Channel Five locally. The Britannica Compton Yearbook, and at least one textbook also plan to publish parts of the story. A conservative estimate by Mr. Grummer of the total coverage to date is at least 10 million people. Unlike many scientific news stories, this one had a minimum of wild speculation, yet stressed the importance of this method of tree propagation as a stepping stone to both the immediate and future uses of the tissue culture technique in tree improvement and cell culture.

Trees are important throughout the world for food products, horticulture and forestry. Forestry is a broad term that includes managing trees for any natural product such as oils, gums, rubber and extracts, as well as for a wide variety of wood products. In the Lake States region, several aspen species are harvested as "popal" and constitute an important source of pulpwood for the paper industry, especially in mixes with longer fibers for specialty papers. A dozen natural stands (or clones) of quaking aspen (Populus tremuloides Michx.) have been found that are larger than other trees in the same area and have three sets of chromosomes in each newly-formed cell, and are called triploids. Normal trees with two sets of chromosomes are called diploids, receiving one set each from the male and female parent during fertilization. The units of inheritance (genes) are transferred from generation to generation on the chromosomes.

Triploid quaking aspen has many pulpwood characteristics of interest to the paper industry (6), but will not root easily from branch cuttings or will easily survive grafting. Sprouts can be grown from roots cut from the parent trees (7), but a more efficient method of obtaining large numbers of trees with the same

genetic makeup is essential for advanced breeding and selection studies. Many other commercially important trees are also difficult to propagate by conventional means of cuttings and grafting; hence, the sudden interest in what may become an efficient means of clonal reproduction from tissue culture. According to Schreiner (8) two of the most important unresolved questions in tree improvement are an efficient method of clonal propagation and forcing of early flowering. Both problems are under study, but we may have the answer to the first question, and we ourselves are watching developments in the second by Pharis, et al. (9).

Perhaps it is not surprising to have received inquiries from dozens of workers around the world who are interested in improving walnut trees, rubber trees, eucalyptus, Douglas-fir, pine, and many other types of trees. As a direct result of the publicity, our laboratory is now under contract with one member company to reproduce a tree of interest to them, and the Institute is entering the preliminary stages for another possible project. We are now in a position to offer member companies practical information for solving some of their wood procurement problems in aspen, and we hope soon to extend this service to other species of importance in other regions of the country.

In this report, the first section will be devoted to a review of the auxin-cytokinin relationship and the possible functions of each hormone in growth. This will be followed by the description of shoot and tree production in aspen tissue cultures, which was submitted for publication to the American Journal of Botany. Parts were also presented at the XI International Botanical Congress at Seattle, Washington, on August 31, 1969, as paper 1174. The last section will cover the effects of amino acid and nucleotide supplements on tissue growth and shoot initiation, and will be submitted later for publication.

We are now at a new plateau of knowledge of tissue culture of aspen, but have not finished the course. The main objective of Project 2351 remains the reproduction of trees from single cells. We now plan to divide our attention three ways between perfecting clonal propagation by rooting shoots grown on callus tissue, increasing the rooting of shoots on the callus to form complete plants, and developing techniques to isolate and grow cells of aspen. The purpose of emphasizing the recovery of complete plants on callus is to develop a balanced nutrient medium that will stimulate single cells to develop as embryoids and grow directly into plants. The alternative would be to grow cells into callus tissue first, which in turn would have to grow shoots. In the long run, the growth studies on nutrient improvement will be the most important to achieve our goal, but for now the reproduction of trees from callus tissue by rooted cuttings is an intermediate point with immediate applications to tree improvement.

A proposal was recently submitted to the Pioneering Research Committee, to continue its support of the Tissue Culture Program, so that we can pursue both the immediate applications, as well as basic studies in cell culture which will ultimately yield the broadest basic answers to problems in tree morphogenesis, physiology, and biochemistry. This proposal is included at the end of this report as a description of future plans.

AUXIN-CYTOKININ RELATIONSHIPS

Auxins and cytokinins are classes of growth hormones produced in plants that stimulate different functions of growth. The natural auxin produced in plants is indoleacetic acid (IAA), and has been studied extensively for its ability to promote cell enlargement and elongation. Light destroys auxin, causing shady parts of plants with more auxin to elongate faster than sunny parts, producing the characteristic bending toward the light observed in crowded plant populations. Auxins also cause rooting, apical dominance, and many other processes explained in most textbooks on plant physiology (10), probably by their influence on cell wall extension, protein synthesis, and general catalytic behavior. A man-made defoliant, 2,4-dichlorophenoxyacetic acid (2,4-D), acts like an auxin at very low concentrations. In fact, its killing ability is based on increasing leaf growth to such a rapid rate that death of the plant comes from depletion of food reserves.

The exact biochemical function of auxin is still unknown (11) but workers in the field now speak of repression or derepression on the operon of the gene. This means they still don't know, but believe that auxin unlocks, or turns on certain genes that otherwise would not initiate the synthesis of amino acids to make proteins, that make specific enzymes for further specific metabolic reactions.

Cytokinins have been studied since 1955, with the isolation of kinetin by Miller, et al. (12). Kinetin promoted cell division in tobacco tissue cultures, and was called 6-furfurylaminopurine. Other N^6-substituted adenines were discovered and synthesized, such as benzyladenine, now called benzyladenine (BA) or 6-benzylaminopurine (BAP). Skoog, et al. (13) recently tested sixty-nine substances which he originally termed cytokinins (to avoid confusion with a group of peptides

called kinins found in animals). Cytokinins stimulate cell division, rather than cell enlargement attributed to the auxins, but the curtain of ignorance has been rent somewhat with the breaking of the genetic code. The code is expressed in the linear order of the gene molecules, in the arrangement of the purine-pyrimidine bases, or the "steps" that bridge the two helical backbones of deoxyribonucleic acid (DNA) that makes up the chromosomes. When the two DNA strands separate and replicate, the order of bases is transcribed to ribonucleic acid (RNA) then to messenger-RNA. This information is then carried to the cytoplasm of the cell as the blueprint for attaching amino acids in the correct sequence, to produce a protein that becomes an enzyme. The messages are formed by the sequence of three bases at a time, where each triplet can specify the attachment of an amino acid or have some other message such as "stop."

Nucleotides found in DNA of the chromosomes contain one of four bases. The two with double rings (purines) are adenine and guanine, and the single-ringed pyrimidines are thymine and cytosine. In RNA, thymine is replaced by another pyrimidine called uracil (14). Skoog, et al. (15) now believe that cytokinins increase the efficiency of binding amino acids of the transcription-RNA to the messenger-RNA from the tRNA pool. He calls this process "modulation of protein biosynthesis."

However, 14 years ago, all that was known about cytokinins was that when the ratio of auxin/cytokinin favored a high concentration of auxin, only roots were produced on callus tissue, but when cytokinin was at a high level, then shoots were produced (12). A balance between the two hormones was necessary for continuous tissue growth. This same phenomenon has been observed in tissue cultures for many plants, and seems to work generally, although exceptions have

been reported. The synergistic relation between kinetin and 2,4-D has been recognized for some time, but only in recent years have physiologists accepted the idea that shoot initiation is mainly dependent upon cytokinin, and that auxin inhibits shoot production.

In our work, a defined agar nutrient medium was used that supplies essential major and minor elements, sugar, vitamins and iron at pH 5.8. The auxin/cytokinin balance was adjusted for either continuous culture or shoot initiation. We reasoned that once shoots were produced, they should make their own auxin that would migrate to the base of the shoot and there stimulate root growth. Shoots were therefore initiated without auxin in the medium, giving a high relative concentration of cytokinin. In our case, we substituted the kinetin in the stock medium with a new cytokinin, 6-benzylaminopurine (BAP), which has been shown to be more active in producing shoots. However, the most active cytokinin for many herbaceous plants, 6-(γ,γ-dimethylallylamino)purine, did not produce shoots on aspen tissue (1).

SHOOT AND TREE PRODUCTION FROM ASPEN TISSUE CULTURES

Clonal propagation is essential for sophisticated breeding programs, but trees vary in their ability to root, sprout or be grafted. Quaking aspen will not root easily from cut branches, but is an important pulp species in the Lake States region (6). Tissue culture may offer a new method of vegetative propagation (16), in addition to current uses to study the morphology, physiology and biochemistry of trees for food, horticulture or forestry (17-19). Reproduction from cells or tissue culture has been reported for several herbaceous angiosperms (20-22), but not for any tree species.

Leafy shoots and roots were initiated on the same pieces of subcultured tissue from European aspen and birch by Jacquiot (23-24), and from triploid quaking aspen by Mathes (3), Winton (4) and Wolter (5). Wolter (5) and Mathes[a] rooted aspen shoots after they were separated from the callus, but none have elongated more than a few centimeters[b]. Winton (1) reported four complete plants grown on aspen callus in 1967, but they all died shortly after transfer to soil, apparently from dehydration[a]. Aneja and Atal (2) also reported rooted plantlets produced on cultured lignotuber tissue of Eucalyptus citriodora Hook., and an undergraduate student of Hackett[a,c] obtained roots and shoots on callus cultured from embryonic root tissue of Ginkgo biloba L.

This paper reports additional shoot initiation from cultured aspen tissue, and describes the rooting of shoots before and after separation from the callus and the growth of plants into normal trees.

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[a] Unpublished.

[b] Personal communication.

[c] Dr. Wesley Hackett directed this student project in the Dept. Agr. Sci., Univ. California, Los Angeles. He is presently at the Univ. California, Davis.

MATERIALS AND METHODS

From one triploid clone (T-2-56) of normally diploid quaking aspen (Populus tremuloides Michx.), root sprouts were grown and callus tissue initiated by methods previously described by Mathes (25) and Winton (26-28). Callus was initiated 1/23/67, and subcultured every 3-4 weeks on Wolter and Skoog (29) defined medium, lacking ammonium nitrate and having the amounts of calcium and potassium nitrate interchanged for more uniform growth of firm white stock tissue (26). Medium 1 contained both an auxin and cytokinin in the amounts of 0.04 mg./l. 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.0 mg./l. kinetin, respectively. The pH after autoclaving was 5.8.

After 12 months, four complete plants were produced (1), and the original stock tissue was subcultured for another ten months on medium 1 with or without 10 mg./l. each of the supplements listed in Table I.

Four weeks after subculture, 20-30 3-mm. cubes (30-60 mg.) from each stock tissue were distributed at two per 125-ml. Erlenmeyer flask or at five per Petri dish, containing 30 ml. of medium 1 without 2,4-D and kinetin (basic medium BM), but having various concentrations of 6-benzylaminopurine (BAP). Shoots were initiated in the dark at 28°C. within a few weeks, and were transferred to an auxin medium or to soil and placed in low (300 ft.-c.) then high (3100 ft.-c.) intensities of tungsten-fluorescent light, and covered with inverted glass dishes until root and shoot growth were established.

TABLE I

COMPONENTS OF MEDIUM 1 AND SUPPLEMENTS (MG./L.)

Medium 1[a]

$MgSO_4$	764	$MnSO_4$	9
Na_2SO_4	425	$ZnSO_4$	3.2
KNO_3	425	H_2BO_3	3.2
$Ca(NO_3)_2$	170	KI	1.6
KCl	140	Thiamine	0.1
NaH_2PO_4	34	2,4-D	0.04
Fe (EDTA)	5.5	Kinetin	1.0
Inositol	100		
Nicotinic acid	0.5	Sucrose	2%
Pyridoxine	0.1	Agar	0.8%

L-Amino acid, Vitamin, and Nucleotide Supplements[b]

AMINO ACIDS

Group 1

- Alanine
- Asparagine
- Aspartic acid
- Citrulline
- Cysteine
- Glutamic acid
- Glycine
- Histidine
- Hydroxyproline
- Isoleucine
- Proline
- Serine

Group 2

- Leucine
- Lysine
- Methionine
- Phenylalanine
- Threonine
- Tyrosine
- Valine

Group 3

- Arginine
- Ornithine

Group 7

- Tryptophan

VITAMINS

Group 4

- Folic acid

NUCLEOTIDES

Group 6

- Adenylic acid
- Cytidylic acid
- Guanylic acid
- Thymine
- Uridylic acid

[a] Adapted from Wolter and Skoog (29).

[b] No Group 5.

RESULTS

Tissue Growth

The 28 supplements were divided into six groups (Table I), and wet-weight increments were measured after four weeks' growth on two series of media. One group at a time was added to the control medium 1, then one group at a time was omitted from medium 100 that contained all supplements. Details were presented in Report Eight, Project 2351 (1968), but all supplements stimulated tissue growth, except tryptophan which was often inhibiting.

During the past two years a variable growth pattern was observed for tissue on medium 1. The best growth was during September-December, when the size and quality of inocula reached a peak four weeks after subculture. From January into summer the rate of growth declined, so that passages of 4-5 weeks still produced less tissue than after three weeks in the fall. During August and September the rate of growth increased, as did airborne fungus contamination, but after the first frost in late September contamination decreased until the following spring. The same relative growth pattern was also observed during the past year for tissue grown on supplemented media. Whether this variable growth is due to internal or external stimuli has not yet been determined.

Test 1 - The First Surviving Tree

In September, four weeks after the twenty-first subculture, tissue was transferred from medium 107 (with all supplements except tryptophan) to basic medium containing 0, 0.01, 0.03 or 0.05 mg./l. BAP. For each BAP medium, 60 inocula were distributed among 12 Petri dishes which were then placed in a dark incubator and checked weekly for six weeks. Shoots were not initiated at the two low levels of BAP, but 8 and 12% of the pieces respectively, produced shoots at 0.03 and 0.05 mg./l.

All callus pieces with shoots were transferred to low light on medium 107. The leaves turned green, and after two weeks thick red roots grew from most of the callus pieces. One shoot grew a root directly from its base and was separated from the callus one month later. The complete plant was left on medium 107 for one month (Fig. 1) under low light in a humid chamber (made by adding water to an empty desiccator). It was then planted in soil sterilized for seven minutes (30) for another month (Fig. 2), and was 10 cm. tall (Fig. 3) when placed under high light intensity in the growth chamber described by Einspahr (31). Five of its nine leaves had been lost from necrosis, but rapid growth began within one week, displaying typical large leaves of vigorous juvenile aspen (Fig. 4). The tree was 46-cm. tall after three weeks and 125 cm. after three months in the growth chamber, and formed its third apical bud when transferred to the greenhouse in May (Fig. 5). Tree I (Table II) was planted in the lawn of the Institute in July, and 1% gibberellin added to the top leaf in a lanoline paste to break dormancy. On July 20, 1969, growth resumed with normal-sized foliage and the lanoline leaf was removed.

Periodic leaf squashes have shown constant triploid chromosome numbers of $3n = 57$ ($n = 19$), identical with the parental clone. Figure 6 shows three


1
2
3
4
5
6
7
A
B
C

sets of chromosomes in a leaf cell prepared by a propiono-orcein squash technique (32), and photographed with a phase-contrast Zeiss Photomicroscope.

CAPTIONS FOR FIGURES 1-7 (See Page 14)

Figure 1. Complete Plant One Month After Separation from Callus

Figure 2. Planted in Soil at Three Months of Age

Figure 3. Transfer of Plant to Growth Chamber when 10 cm. Tall

Figure 4. Vigorous Growth After Three Weeks when 46 cm. Tall

Figure 5. Transfer to Greenhouse After Three Months in Growth Chamber when 125 cm. Tall

Figure 6. Triploid Leaf Cell from Tree I (3n = 57 Chromosomes)

Figure 7. Stock Tissue of Triploid Quaking Aspen Grown for Eight Weeks from Subculture on Media 1 (7A), 100 (7B) and 107 (7C)

TABLE II

GROWTH OF TREE I FROM TISSUE CULTURE

	Date	Height, cm. Shoot	Root	Elapsed Time Days	Months	
Subcultured tissue 107 to BAP-.05	9-23-68			0		
Shoot initiated	10-14-68	0.3		21		
Medium 107, 300 ft.-c.	10-17-68	0.4		24		
Root initiated from shoot	11-1- 68	0.5	0.2	39	1.5	
Plant separated from callus	11-26-68	0.8	2.0	64	2.5	(Fig. 1)
Planted in soil, 300 ft.-c.	12-30-68	2.5	7	98	3	(Fig. 2)
Growth chamber, 3100 ft.-c.	2-11-69	10	-	141	5	
After 3 weeks, vigorous growth	3-4- 69	46	-	162	5.5	(Fig. 3)
After 7 weeks, first bud	4-8- 69	71	-	197	7	
Second flush	5-2- 69	105	-	221	7.5	
Greenhouse, third bud	5-20-69	135	-	239	8	(Fig. 4)
Planted at Institute	7-11-69	122	-	291	9.5	
Resumed normal growth	7-20-69	123	-	300	10	

Test 2 - Shoot Initiation

In October, tissue from media 1, 100 and 107 was distributed among ten flasks each, containing medium BAP-.05 or BAP-.15. Some stock tissue was left to grow for an additional four weeks, and shows the relative growth rates on the three stock media in Fig. 7.

In the shoot initiation test, the dark incubators were opened weekly for seven weeks for inspection, and the rate of shoot initiation is shown in Fig. 8. Shoots, still attached to the callus, were transferred to low light and medium 107 on the same day or one week after their discovery, and turned green within a few days. However, when left attached to the callus, shoots did not elongate and usually died after a few months. The original tissue did not grow but remained white on inocula having shoots, but a few pieces with only roots turned brown. On 20-70% of the inocula, one or two vigorous shoots per piece usually appeared within 2-4 weeks and grew to 1-3 cm. after transfer to low light (Fig. 9B), but the vigor of the emerging shoots declined when initiated after four weeks. Two shoots rooted in the light before they were separated from the callus. Tree III, that survived (Fig. 9C), grew from tissue transferred from medium 107 to BAP-.05 (Table III), but the one that died grew from tissue 1 and had abnormal leaves with frilly margins.

On medium BAP-.15, 85-95% of the inocula initiated 5-10 shoots each (2-5 mm. tall) within 3-7 weeks (Fig. 9A), and firm white tissue of the inocula doubled in size. None of the shoots rooted before separation from the callus, but Trees IV and VI (Table III) grew from two shoots cut from the same piece of callus and rooted in medium 107 (rooted cuttings).

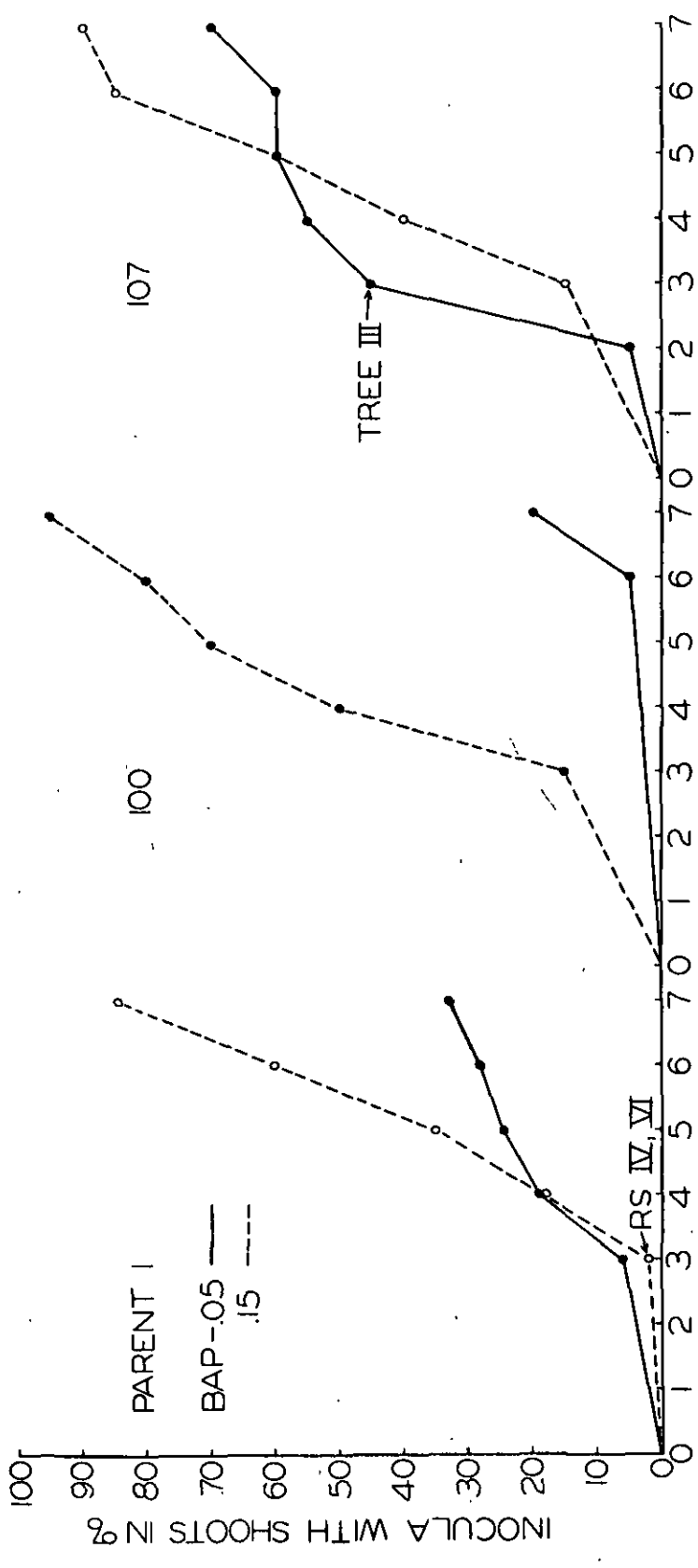

Figure 8. Rate of Shoot Initiation on Inocula Cut from Tissues 1, 100 and 107 Four Weeks After Subculture, and Transferred to Media BAP-.05 or BAP-.15. Cumulative Percentages are Given of Inocula with Shoots for Each Subsequent Week After Transfer

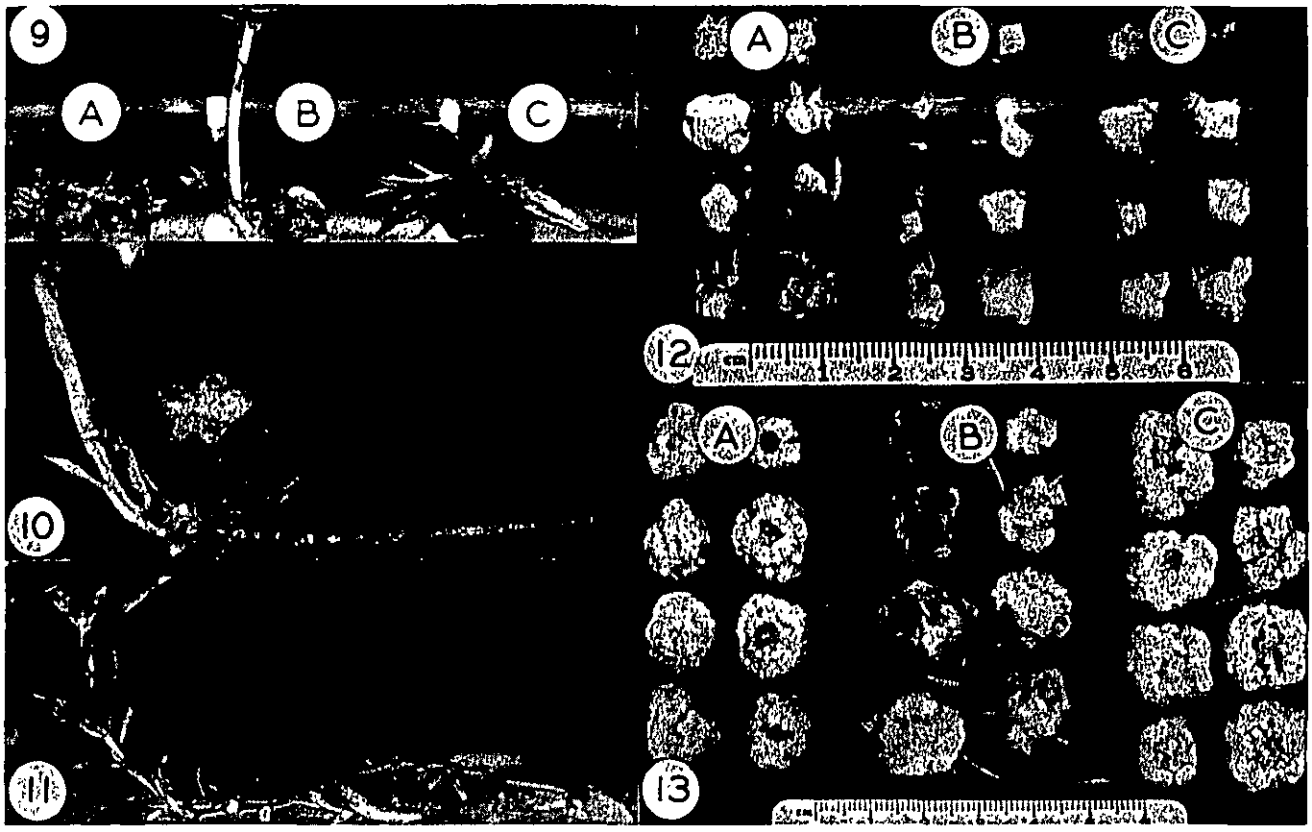

Figure 9. Initiation of Stunted Shoots on Medium BAP-.15 (9A) and Vigorous Shoots on Medium BAP-.05 Without Root (9B) or Rooted (9C) While on the Callus on Auxin Medium. The Complete Plant (9C) is Tree III

Figure 10. Shoot Rooted in the Dark on Medium BAP-.05

Figure 11. Tree II Separated from the Callus and Growing on the Surface of Medium 107 in the Light

Figure 12. Shoot Initiation on Medium BAP-.05, on Inocula Cut from Tissues 1 (12A), 100 (12B) and 107 (12C)

Figure 13. Growth of Stock Tissues Four Weeks After Subculture to Media 1 (13A), 100 (13B) and 107 (13C). Note the Partial Friability (Tendency to Crumble) and Rooting of Tissue 100 Which Occurred only During this one Passage in November

TABLE III

ASPEN TREES GROWN FROM COMPLETE PLANTS AND ROOTED CUTTINGS (RC)
INITIATED ON CALLUS TISSUE IN 1969

Test	Subculture Date	Parent[a] Medium	Vessel[b]	BAP	Weeks After Subculture						Tree		
					Shoot	Light-107[c]	Root	Separated	Soil	GC[d]	Plant	Frequency[e], %	Height, cm. Aug
1	9-24	107	D	.05	3	4	6	10	15	23	I	12	130
2	10-21	107	F	.05	3	5	3	7	9	13	III	5	149
		1		.05	4	5	7	Died	-	-	-	-	-
				.15	4	5	14	8	14	17	IV RC[f]	-	82
					4	5	12	8	12	17	VI RC	-	76
3	11-18	1	$\bar{D}$	05	4	5	4	6	9	12	II	2	127
					3	5	9	Died	-	-	-	-	-
4	11-25	100	D	05	4	5	9	Died	8	-	- RC	-	-
5	12-23	1	F	.05	4	7	5	19	19	19	IX	5	52

[a]All inocula were cut four weeks after subculture, except two weeks for Test 5

[b]Vessel: D-Petri Dish, $\bar{D}$-Dish sealed with Saran, F-125-ml. Erlenmeyer flask covered with foil.

[c]Low light (300 ft.-c.) on medium 107.

[d]Growth chamber (3100 ft.-c.).

[e]Ratio of the number of trees/inocula of the same treatment.

[f]RC = rooted cutting.

Generally, the number of inocula that produced stunted shoots was uniformly high on all three stock tissues transferred to medium BAP-.15. No shoots were initiated before three weeks, but their appearance increased thereafter until the end of seven weeks. Conversely, the number of inocula with shoots was lower on medium BAP-.05, and varied according to the tissue. Shoot initiation began rapidly on tissue 107 after two weeks then leveled off, but did not start until three and six weeks, respectively, on tissue 1 and 100, and never reached the high frequency found on all tissues placed on medium BAP-.15. This same response was reported earlier by Winton (1) between BAP at 0.05 and 0.15 mg./l. on tissue from medium 1, confirming Wolter's (5) optimum concentration for shoot production at 0.14 mg./l. BAP.

Test 3 - Sealed Versus Unsealed Dishes

In November, tissue from media 1, 100 and 107 was subcultured to medium BAP-.05. Thirteen dishes of each tissue were sealed in clear Saran wrap and placed in the dark incubator with the other half of the dishes left unwrapped. After two weeks the Saran was removed, the number of pieces with roots or shoots was recorded and all dishes were returned to the incubator unwrapped and examined again at the end of three weeks.

Table IV shows significant shoot initiation only from tissue 1 on medium BAP-.05. After two weeks, not as many shoots were found in sealed as in unsealed dishes. But after three weeks, 45 and 52% respectively, of the pieces had shoots in unsealed dishes and dishes sealed during the first two weeks, which may not be a significant difference. However, shoots in dishes sealed for two weeks were more vigorous (taller) and may indicate the presence of a volatile growth stimulator, such as carbon dioxide postulated by Street (33) from his cell suspension studies or ethylene, reported in suspensions of several herbaceous species by Gamborg and LaRue (34).

TABLE IV

SHOOT INITIATION IN TEST 3, AFTER TWO AND THREE WEEKS ON MEDIUM BAP-.05 IN SEALED (S) OR UNSEALED (U) DISHES

Stock Tissue	Dishes[a]	Percentage of Inocula After 2 and 3 Weeks with Shoots		Roots		Dead	
1	S	14	52[b]	4	6	0	0
	U	22	45	2	5	0	0
100	S	0	6	0	8	25	32
	U	0	5	0	0	29	49
107	S	0	0	0	3	6	22
	U	2	3	6	2	2	15

[a]Sealed with Saran wrap.

[b]Tree II initiated after four weeks.

One shoot rooted on medium BAP-.05 after four weeks in the dark (Fig. 10) in a dish that had been sealed, and was separated from its callus one week after it was discovered and transferred to low light on medium 107 (Fig. 11). Four weeks later, when the shoot was 13 mm. and the root 40-mm. long, Tree II was planted in soil in the growth chamber. Another shoot rooted after four weeks in the light on medium 107, but died before separation from the callus.

In this test, tissue 1 more than doubled its rate of shoot initiation from Test 2 the previous month, i.e., from 20 to about 50% after four weeks on medium BAP-.05. At the same time, tissue 107 dropped from 55% in October to 3% in November, and tissue 100 increased slightly from zero to 5-6%.

Inocula from each tissue, with and without shoots, are shown in Fig. 12 after four weeks in the dark on medium BAP-.05. Figure 13 shows inocula from each

stock tissue maintained on media 1, 100 or 107 for the same period, or after four weeks of the twenty-third passage. Average wet weights were 521 ± 121 mg. for tissue 1, 675 ± 279 mg. for tissue 100 and 755 ± 272 mg. for tissue 107. The high percentage of rooted and dead pieces of tissue 100 on BAP-.05 was correlated with the same twenty-third passage of stock tissue 100, which was also the only passage in which some tissue became friable and tan and had a high percentage of rooting from the callus. Tissue 107 also had some rooting and necrosis on BAP, but the stock tissue remained firm and white.

Test 4 – The Number of Inocula Per Dish

Inocula from tissue 100 and 107 were also distributed among dishes 20-mm. deep containing medium BAP-.05. Four dishes of each tissue contained 1, 2, 5 or 10 inocula. Two dishes of each tissue were sealed with Saran and two left unsealed and all placed in a dark incubator for six weeks. The number of inocula with new shoots were recorded weekly, but those with roots were counted once at the end of six weeks.

Figure 14 shows no apparent difference in shoot initiation between sealed and unsealed dishes (verifying the same effect reported in Test 3), or between 2, 5 or 10 inocula per dish. However, shoot production was higher on tissue 100 than 107. In the two sealed dishes of tissue 100 with a single inoculum, one had a shoot 2.5-cm. tall and one root, and the other had two shoots 1.5-cm. tall. Vigorous shoots were also found occasionally in dishes with more than one piece; however, five inocula per dish appears to be the most efficient for producing the maximum number of the tallest shoots for clonal propagation.

The percentage of inocula with roots after six weeks is shown in Fig. 14 in parentheses. Except for tissue 100 in sealed dishes, there appears to be

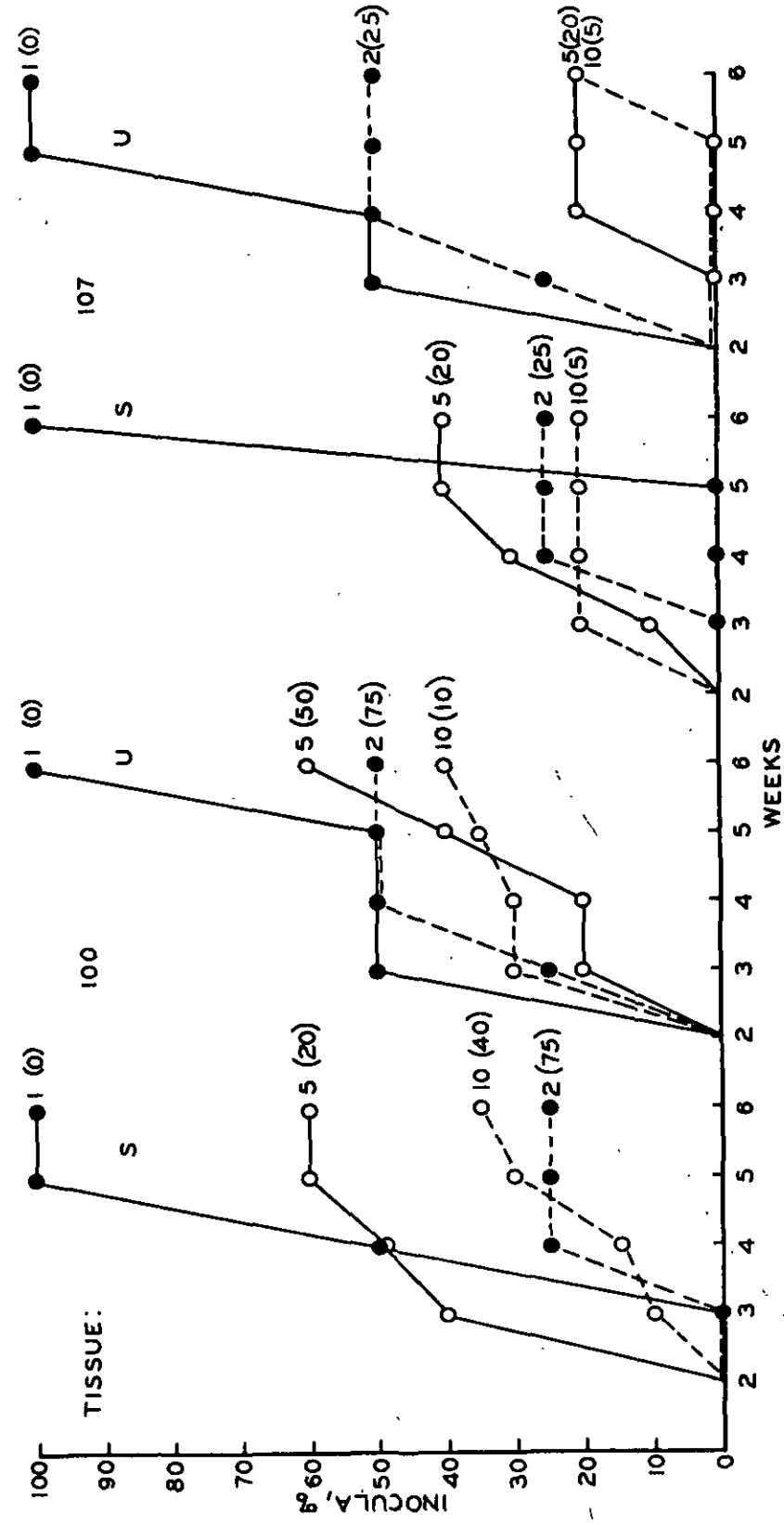

Figure 14. The Rate of Shoot Initiation for Two Tissues on Medium BAP-.05 in Sealed (S) or Unsealed (U) Petri Dishes. The Number of Inocula per Dish is Shown for Each Curve, and the Percentage of Inocula with Roots at the End of Six Weeks is Given in Parenthesis

no correlation between the rate of shoot initiation and total root production, except that no roots formed on single pieces per dish.

Test 5 - Age of Tissue for Inocula

Beginning the second week after subculture, inocula from each tissue were distributed among ten flasks containing medium BAP-.05. Tissue 107 remained firm and white throughout the eight weeks, but tissues 1 and 100 turned brown after four weeks and were discontinued. The number of inocula with new shoots and roots were recorded each week, but were so few that only the totals produced after six weeks are summarized in Fig. 15 for each age of tissue.

The objective of this test was to compare shoot initiation in late November with similar results of Test 2 made one month earlier, in order to relate rooting with shoot initiation as a possible indicator of endogenous auxin production in older tissue.

For tissue 107, most shoot initiation occurred on inocula cut from stock tissue three weeks after subculture and placed on medium BAP-.05. Shoot production dropped sharply on inocula cut after four weeks, just when root initiation from callus took an opposite upward swing. Figure 15 shows a possible shoot-root relationship for tissue 100, where endogenous auxin production would explain the rise of root initiation and the fall of shoot production in tissue left on stock medium longer than four weeks before transfer to BAP. For tissue 100, shoot production rose from zero to 15% and rooting dropped from 25 to 5% on inocula cut from stock tissue, respectively, three and four weeks after subculture. The same type of reversal (between rooting and shoot initiation) observed for tissue 100, cut when three and four weeks old, also occurred for tissue 107 when one week older and for tissue 1 when one week younger (Fig. 15).

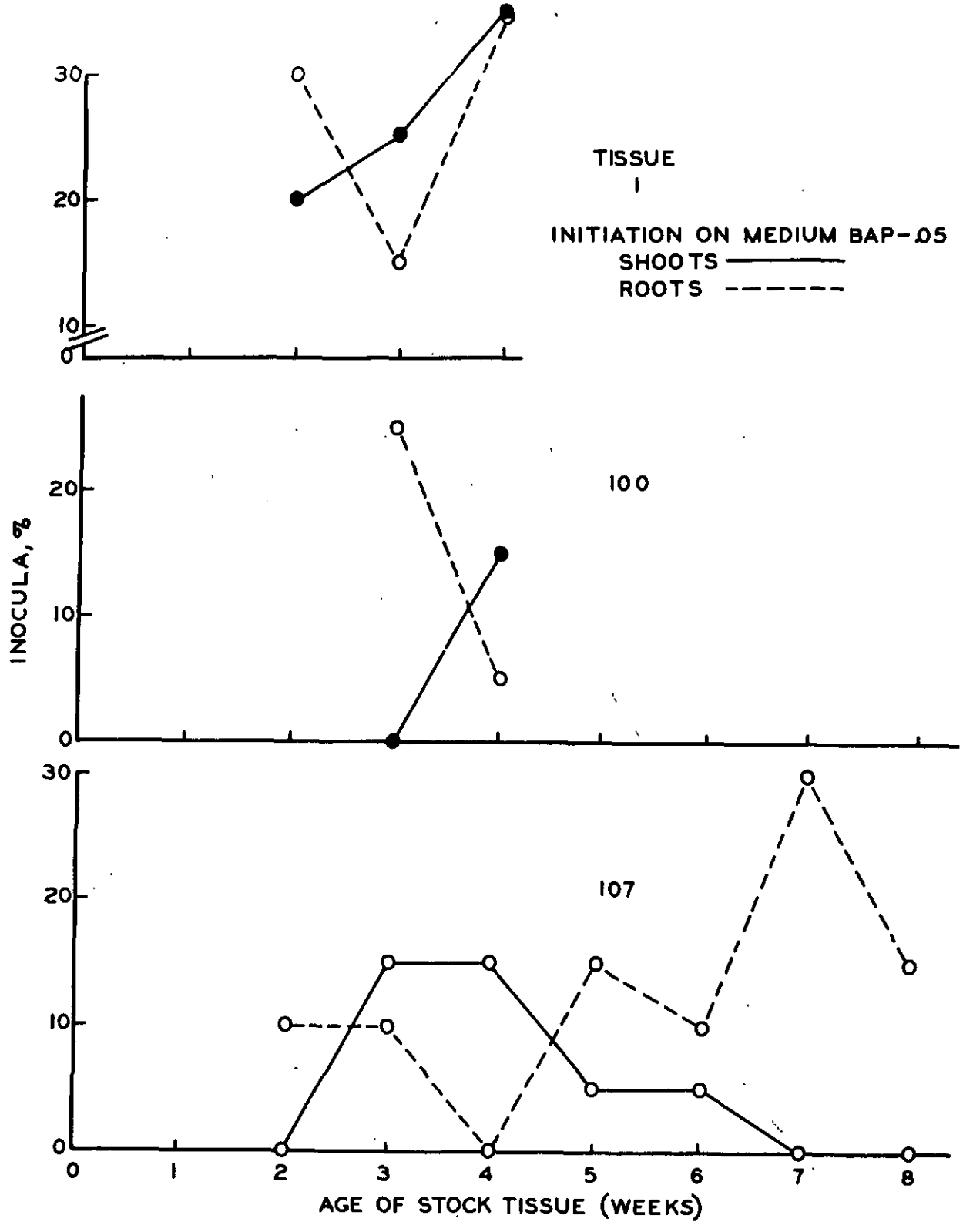

Figure 15. The Percentage of Inocula with Roots or Shoots, Cut from Three Tissues on Successive Weeks After Subculture and Transferred to Medium BAP-.05. Stock Tissues 1 and 100 were not Used for Inocula After Four Weeks of Growth. Each Point is a Six Week Accumulation from the Time of Transfer.

Comparing four week old tissue in this test with Test 2, Fig. 8 and 15 show that after six weeks, shoot initiation dropped from 70 to 15% between October and November for tissue 107. However, the frequency rose from 5 to 15% for tissue 100, and from 30 to 35% for tissue 1. The same relative changes were also observed among stock cultures. Apparently, each stock tissue underwent a different metabolic change during October-November, changing the relative rates of shoot and root initiation on inocula placed on BAP media. Tissue 107 produced the most shoots in September-October and tissue 1 did best in November-December.

In subsequent tests, organogenesis dropped to near zero for all tissues during late winter and spring. During the summer of 1969, some shoots grew from tissue 100 on medium BAP-.05, and one rooted on the callus but died after separation. In late summer, shoots on all tissues were more abundant on BAP-.15 than on BAP-.05.

Rooting of Cuttings

Although the objective was to obtain complete plants on callus, some shoots were cut during several experiments and rooted in auxin media or in soil under low light.

Vigorous shoots, cut from inocula on medium BAP-.05, grew new callus but no roots from their base. On the other hand, stunted shoots never rooted inocula on medium BAP-.15, but had little difficulty rooting after they were separated from the callus and placed in soil or medium.

DISCUSSION

These tests demonstrated that at least one tree species can be reproduced from tissue culture, apparently without adverse effects from the undifferentiated phase of cell growth. One and two-year old tissue of triploid

quaking aspen remained firm and white when subcultured to fresh stock media every four weeks. All supplements tested stimulated the growth of stock tissues and caused an increase in shoot initiation, except tryptophan that often inhibited growth and shoot initiation to varying degrees, depending upon the time of year.

Previous results (1) were substantiated, that a few vigorous shoots were initiated on medium BAP-.05, and only these grew roots and became complete plants on the callus. However, numerous stunted shoots grew on medium BAP-.15 and only rooted after separation from the callus, verifying Wolter's (5) original work.

An increase in frequency of plant production would permit a new method of studying morphogenesis in vitro, from earliest differentiation to seedling and tree stages. Rooted cuttings, however, offer the best method at this time for mass clonal propagation of aspen species. Stock cultures appear stable on defined media, but only long-range experiments will show for how long the genotype will remain unchanged in cultures of undifferentiated tissue. Haploid, polyploid, and superior diploid trees should now be easily propagated from tissue culture with modifications of these techniques, and eventually even from single cells (36-37).

The elongation of plantlets into trees only occurred on an auxin medium, under a high light intensity (3100 ft.-c.) and high humidity. The lower intensity of 300 ft.-c. was insufficient to promote elongation past a few centimeters, but under both light sources the leaves had to be protected against dehydration until the plant could control its own transpiration. Trees were grown from both complete plants and rooted cuttings.

Perhaps only a partial explanation can be offered now of why others have not yet reproduced trees from tissue cultures. Torrey (35) reviewed several

reports of a progressive loss of organ-forming capacity during prolonged culturing of herbaceous species, and explained the loss by an increase of polyploid tissue in older cultures. Wolter's (5) tissue may have developed polyploidy (during culturing) during the seven years of culturing, and account for the slow emergence of stunted and aberrant shoots on the underside of necrotic tissue. However, the tissue used by Mathes (3) was the same (T-2-56) used in this laboratory, and was also seven years old when normal shoots were separated and rooted. Complete plants were not produced on his callus, but shoots were normal in appearance and had triploid counts when examined in our laboratory in 1967.

One factor for our success may have been the use of one and two-year old cultures. From another faster-growing natural triploid clone (T-38-59) of quaking aspen, shoots were produced from tissue subcultured to medium BAP-.15 during the second passage after isolation, indicating that organogenesis may be a function of the age of the tissue from isolation. In this study, however, the age of tissue after subculture was critical when inocula were cut from tissue grown more than four weeks without subculture and placed on BAP media, rooting increased and shoot initiation decreased, supporting the hypothesis of endogenous auxin production in stock tissue after four weeks. Another problem, the rhythm of tissue growth during successive passages throughout the year is unexplained, but has been observed by other workers and mentioned in private conversations.

In supplemental media, the effect of tryptophan is not always inhibiting and may stimulate both growth and shoot initiation in summer, when cultures without tryptophan grow more slowly and do not produce shoots. In suspension cultures, tryptophan increases cell separation, acting similar to high levels of 2,4-D (unpublished).

In summary, the three most important factors in the survival of plants and rooted cuttings appear to be a combination of (1) auxin medium, (2) high light intensity and (3) high humidity. The absence of any one of these factors has so far inhibited shoot elongation and subsequent tree formation.

EFFECTS OF SUPPLEMENTS ON TISSUE GROWTH AND SHOOT INITIATION

In Report Eight (1968), two tissues were compared that were isolated from the same clone (T-2-56) of triploid quaking aspen on 12/7/66 and 1/23/67. Tissue 12/7/66 was later discarded, after a year of study that showed the superiority of tissue 1/23/67 to grow callus and produce shoots. Both tissues were also tested on media made with or without each of the six groups of supplements shown in Table I. By the end of four passages of four weeks each, the fastest growth of firm white tissue was on medium 107, containing all supplements except tryptophan. The next best growth was on medium 100 with all supplements, providing at once a fortuitous system to study the effects of tryptophan as the precursor of the natural plant auxin 3-indoleacetic acid (IAA) (38). The control tissue on medium 1 grew significantly slower than either of the other two tissues, although all three tissues originated from one selected piece of firm white callus, initiated from one root-sprout segment on medium 1. Tryptophan caused a slight inhibition in growth when added to supplemented media, but the main question was what class of supplements, the amino acids or nucleotides, was responsible for the major growth increase over tissue on medium 1.

During the past nine months, after the shoot initiation tests were completed, new series of experiments were run to determine (1) whether amino acids or nucleotides or both stimulated tissue growth, (2) if either arginine or glutamine could replace the 23 amino acids in the supplemented media 100 and

107, with or without the nucleotides, and (3) what specific substances most influenced callus growth and shoot production among the supplements.

AMINO ACID SUPPLEMENTS

Materials and Methods

Inocula were cut from stock tissues 1 and 107, weighed, and distributed among three dishes for each tissue, at five per dish, for each test medium shown in Table V.

TABLE V

MEDIA FOR AMINO ACID SUPPLEMENT TESTS

Treatment	Medium	Added to Medium 1[a]
1	1	--
2	G	Glutamine
3	G+6	Glutamine + Group 6[b]
4	A	Arginine
5	A+6	Arginine + Group 6
6	6	Group 6
7	100	All supplements[c]

[a] See Table I for composition.

[b] Nucleotide supplements, Table I.

[c] Amino acid, nucleotide and folic acid supplements, Table I.

Note: In medium 100, 10 mg./l. of each of the components are used, therefore 0.23 g. of glutamine or arginine were used to replace the 23 amino acids.

Tissue was grown in a dark and humid incubator at 28°C. for four weeks, then weighed again, photographed, subcultured and the new inocula weighed and placed on fresh media for the second passage. Four sequential passages were grown for both tissues on each media, but the first two passages were four weeks long and the second two were six weeks long.

Results

Parts of the data are presented in three different ways to aid in the interpretation of the results. The average wet weights of the inocula and the growth increment (plus or minus one standard deviation) was calculated for both tissues on all media for each passage, for a total of 58 treatments (tissue 107 was grown on medium 107 as an additional control during the third and fourth passages). Initial and increment averages are plotted in Fig. 16 for tissues 1 and 107 for the first passage, along with the standard deviations. The generally superior vigor of tissue 107 over tissue 1 is evident in the plotting of increments, and some significant differences would probably have shown up on media 3, 4, 5 and 6 between the two tissues if they had been determined. The inocula of tissue 107 were slightly heavier than those of tissue 1, except on the first two media (treatments 1 and 2 in Fig. 16). However, small differences between inocula have not caused large differences in increment in past experiments, although with a few exceptions, inocula weights were the same for each tissue. And, since the main question is performance between media, the two tissues were not directly compared statistically for the same passage, but rather the media were tested separately for each tissue. If, during preparation of the manuscript for publication, comparisons seem important between tissues, the data can then be treated in this manner. Figure 1 is included only to give a feeling for the variations.

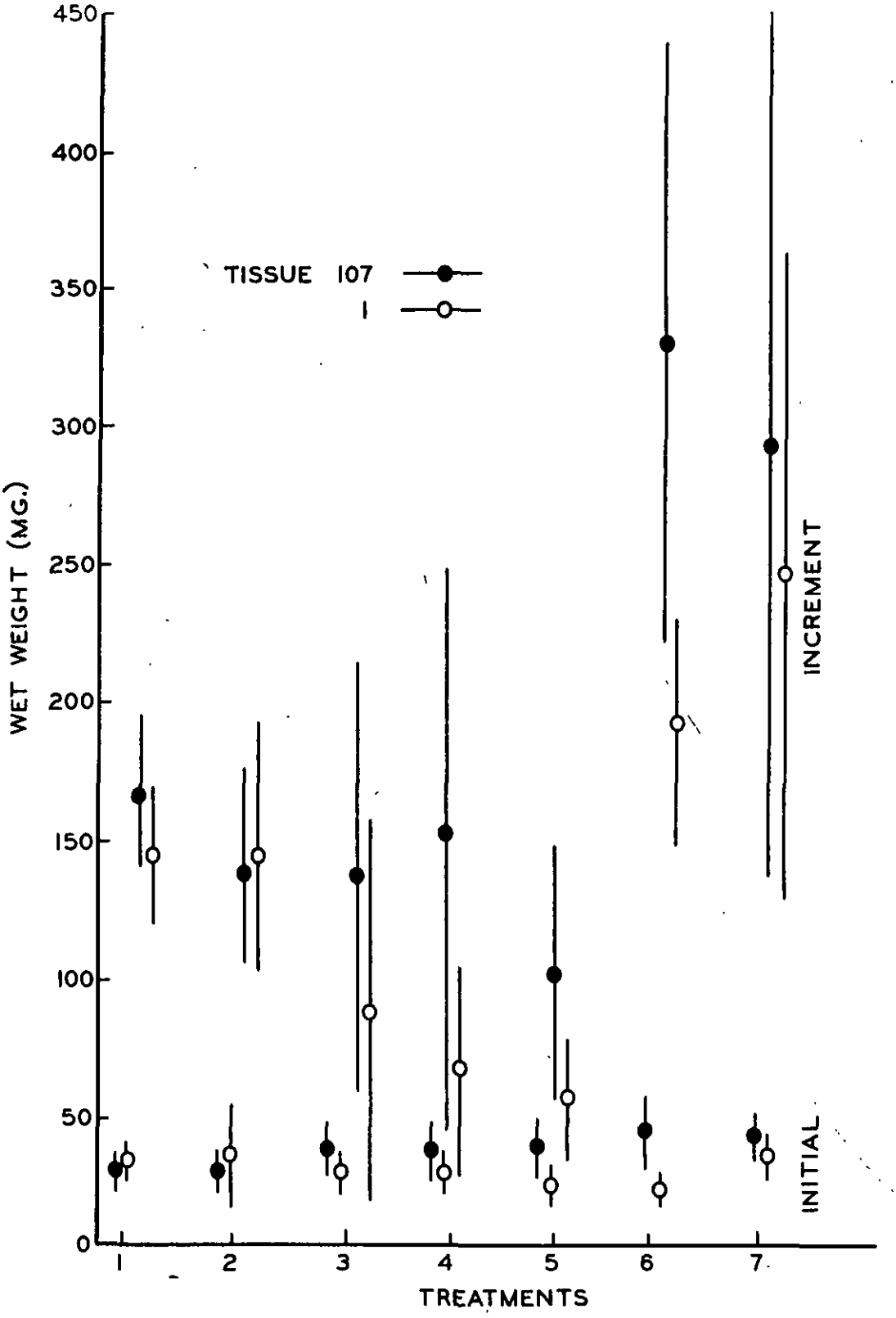

Figure 16. Average Initial and Increment Wet Weight (Plus or Minus One Standard Deviation) of Tissues 1 and 107 at the End of the First Four Week Passage. Treatment Numbers Correspond to Media Described in Table V

In Fig. 17 and 18, the average increments without variations are plotted for tissues 1 and 107, respectively, for each passage. Because the passages were sequential with the same tissues, the growth for each medium is a true graph of performance in time, and shows the relative change in increment on succeeding passages, as well as between passages of different duration. The lines are omitted between the second and third passages (when the duration changed from four to six weeks) merely to avoid confusion, but the increments for media 6 and 100 are put in as broken lines to show the abrupt drop in growth on medium 6 of both tissues after four weeks. Conversely, the sudden growth increase on medium A+6 is associated with a change in the duration of the passage from four to six weeks.

Perhaps the most meaningful comparisons are shown in Table VI, where average increments are arrayed in descending order for each tissue for each passage, and tested against one another by Duncan's Multiple Test (38) at the 5% level of significance. In order to be significant, increments must differ by 20%, which is the usual standard of either experimental or economic importance attached to such tests. Using this standard, the results in Table VI give the clearest answer to the original question of whether glutamine or arginine can replace 23 amino acids, with or without nucleotides, or if the nucleotides alone stimulate growth. And, being biological data, one would not expect a simple answer, nor was one found.

In Table VI, however, two distinct patterns are at once evident for both tissues, perhaps adding reliability to the results. First, in the four-week passages, the callus that grew on medium 6, (medium 1 plus nucleotides) was significantly heavier than on any other test medium, and was only approached or surpassed by tissue on control medium 100. But in the six-week passages, growth

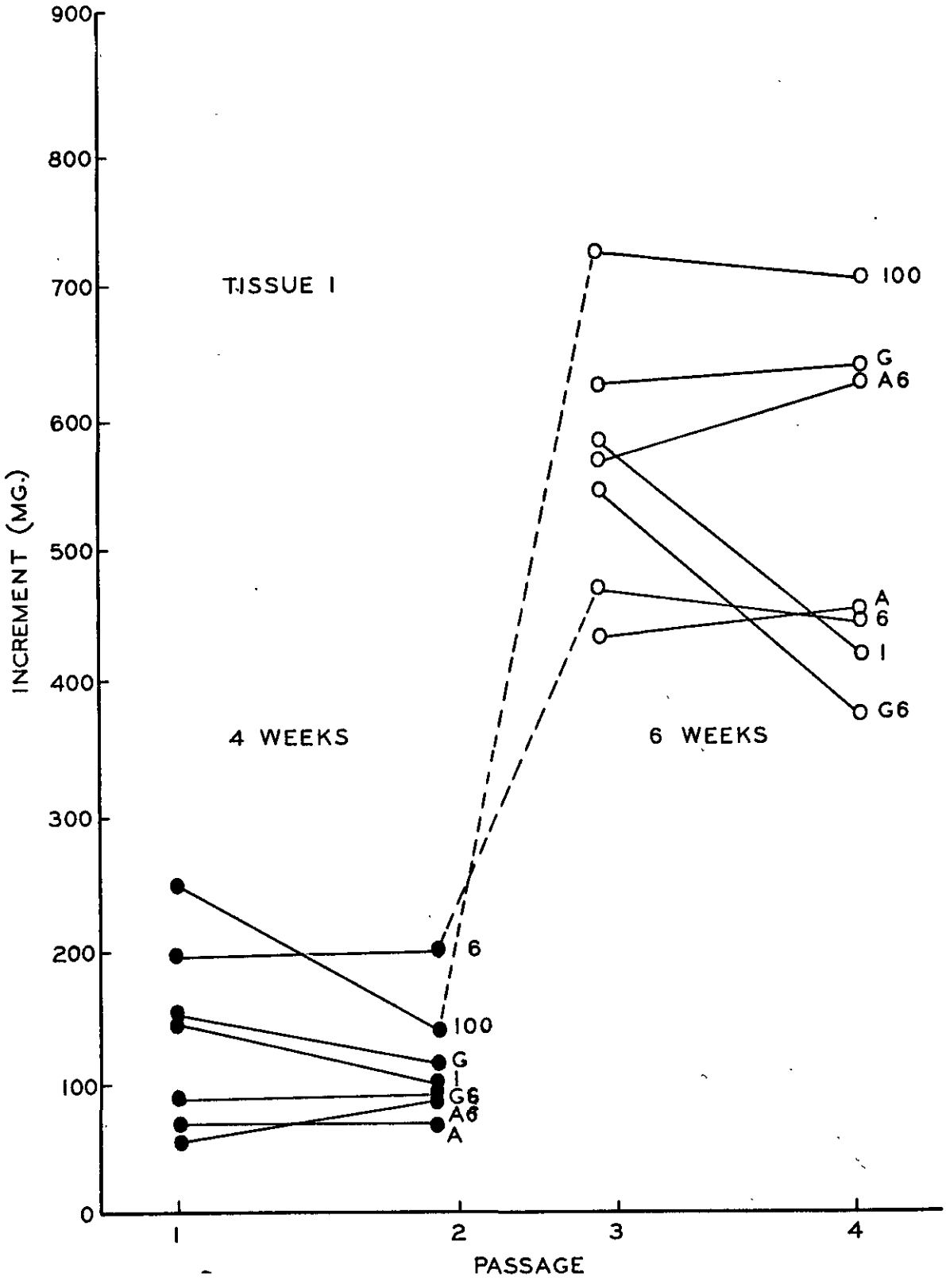

Figure 17. Average Increments of Tissue 1 for Two Passages Each of Four and Six Weeks on Media Described in Table V

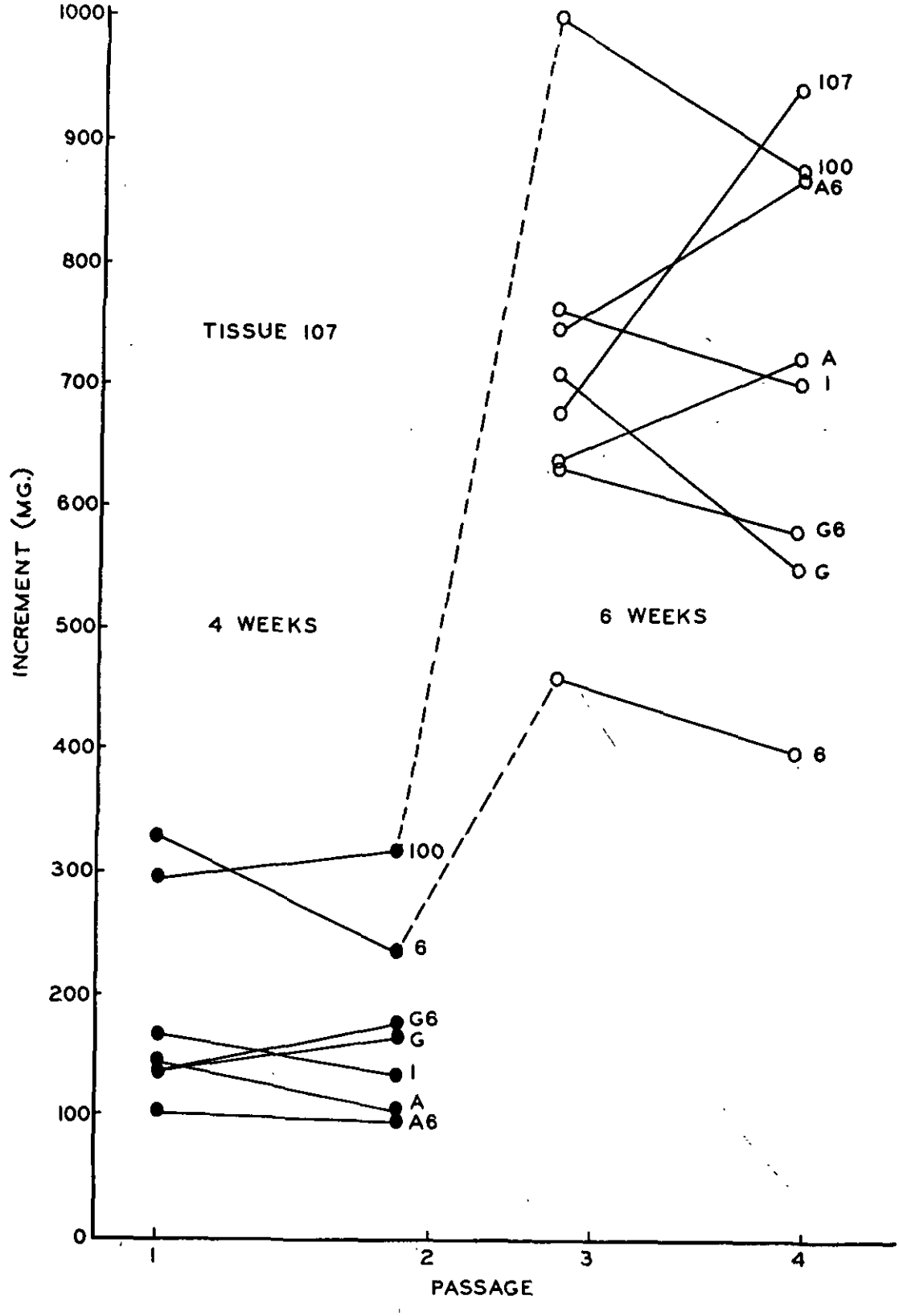

Figure 18. Average Increments of Tissue 107 for Two Passages Each of Four and Six Weeks on Media Described in Table V

TABLE VI

AVERAGE INCREMENTS (MG.) OF TISSUES 1 AND 107 GROWN ON SIX MEDIA FOR FOUR AND SIX-WEEK PASSAGES

Passage[a] 1		2		3		4	
Tissue 1							
100[b]	247[c]	6	194	100	715	100	696
6	193	100	135	G	619	G	630
G	146	G	121	1	572	A6	617
1	145	1	99	A6	562	A	448
G6	89	A6	83	G6	540	6	440
A6	57	G6	82	6	462	1	416
A	68	A	68	A	403	G6	370
Tissue 107							
6	330	100	317	100	996	107	938
100	293	6	232	1	757	100	862
1	168	G6	175	A6	742	A6	862
A	147	G	164	G	706	A	719
G	139	1	131	107	672	1	698
G6	138	A	105	A	634	G6	579
A6	103	A6	96	G6	633	G	548
				6	455	6	396

[a] Passages 1 and 2 = 4 weeks, 3 and 4 = 6 weeks

[b] Treatments included media 1 and 100 as controls, in addition to medium 1 + glutamine (G), or + arginine (A), or + five nucleotides (6) or the combinations of G6 or A6.

[c] Average wet-weight increments connected by lines are not different at the 5% level of significance, according to Duncans multiple test (DMT) (38).

was poorest on medium 6 and enhanced for both tissues on medium 1 plus arginine and the nucleotides of group 6 (A+6). In the longer passages, arginine alone (A) or glutamine plus nucleotides (G+6) were inhibitory when added to medium 1, and glutamine alone added to medium 1 was only superior for tissue 1 which had never been grown with any of the supplements.

To the question then, if the tissue is grown for only four weeks before subculture, the addition of nucleotides will stimulate growth. However, neither glutamine nor arginine, with or without nucleotides, can replace the 23 separate amino acids in medium 100. There is also the possibility that folic acid enhances growth above that which is stimulated by amino acids, but this was not tested. When grown for six weeks, the story is different and nucleotides inhibit growth when added without amino acids. Glutamine (G) alone added to medium 1 is sufficient for tissue 1 to grow faster, but arginine and nucleotides (A+6) were the next best. Which brings up the question of Why?

However, before a judgment is reached based on wet-weight increments, a second criterion, of tissue quality, must be introduced. Not only should tissue be growing at an efficient speed for continuous culture, but the tissue must be firm, white and vigorous. In reality, we have for many years selected callus during the early period of initiation and establishment, on the basis of firm white tissue rather than for rapid growth alone. Often the fastest growing callus was also friable (crumbly) and yellow, and never differentiated into plant organs of roots or shoots. During the past two years, our studies with nutrient improvement have stressed the rapid growth of firm white tissue.

In this study, the two best pieces of callus with firm white tissue were selected from each medium, at the end of the second and fourth passages, and

photographed. Only tissue 107 is shown in Fig. 19, because all of tissue 1 (except that growing on medium 107) was about equally unimpressive for quality and had less firm white tissue than did tissue 107. After four weeks, the best tissue quality on any of the test media was far inferior to that on medium 107.

At the end of the fourth passage, however, or after the second 6-week passage, the growth of firm white tissue was impressive on all media for both tissues. Figure 20 shows the relative vigor of tissue 1 and tissue 107 on the same medium. But more important, it also shows that both the quality and amount of firm white tissue on medium A+6 is about equally as good as on medium G for tissue 1 (Fig. 20 top), and is superior to tissue on any other test medium for tissue 107 (Fig. 20 bottom). Fortunately, the visual observation on quality is consistent with the criterion of excellence based on growth increment.

Discussion

In Test 5 of this report, shoots were initiated easily on inocula cut from stock tissue that was four weeks old or younger from subculture, but fewer shoots and more roots appeared on inocula from tissue older than four weeks. The hypothesis was that after four weeks the tissue either began to make auxin or obtained a threshhold concentration within the plant. This endogenous auxin production inhibited subsequent shoot initiation on inocula cut from that tissue and stimulating root production on BAP media. The results of this study shed more light on this hypothesis, and point to several areas of future investigation.

The appearance of a critical level of auxin between the fourth and fifth weeks after subculture could be the response of the genes to a feedback system, from a build up of metabolic products or to some other unknown trigger mechanism.

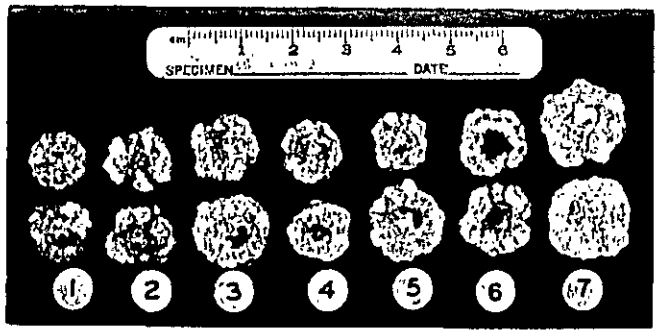

Figure 19. Tissue 107 After the Second Four-Week Passage on Seven Treatments (Media) Described in Table V

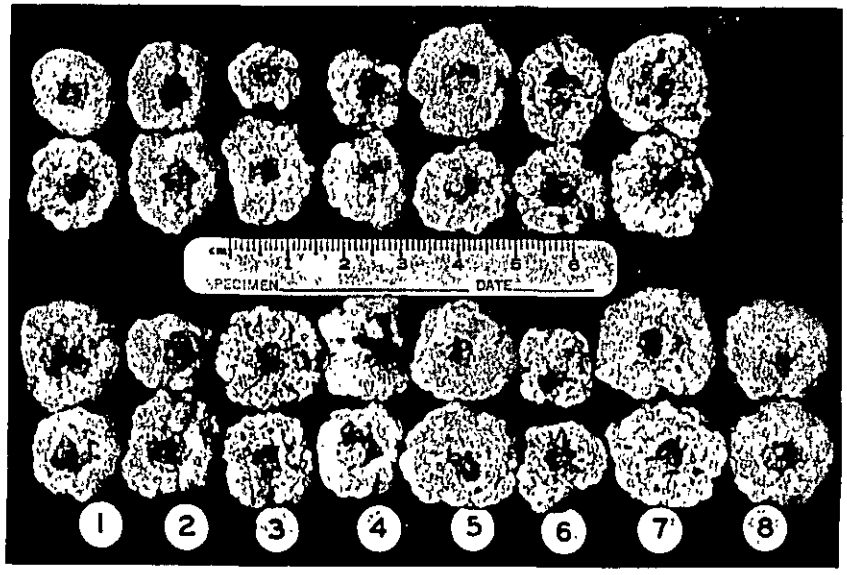

Figure 20. Tissue 1 (Top) and 107 (Bottom) After the Second Six-Week Passage on Seven Treatments (Media) Described in Table V. Tissue 107 was also Grown on Medium 107 as a Second Control

An assumption is made that somehow auxin production is triggered, and the effects are those observed during the past year of a reversal of shoot-root production. In this study, an apparent change observed in the nutrient requirements from nucleotides to amino acids might be the reason for the shoot-root reversal.

In another earlier section, the relationship between auxin and cytokinin was discussed, with a general statement that cytokinins aid in the transfer of genetic information out of the nucleus to where enzyme proteins are made. (In normal plants, genetic transfer by RNA would also include cell differentiation, but that process is blocked in stock tissue cultures that maintain an essentially undifferentiated tissue.) Nucleotides are used as a source of building blocks for both DNA and RNA during cell division and information transfer, and amino acids are used later in the sequence as building blocks for enzymes and other proteins of cell growth.

So, in a rough sense, nucleotides are associated more with cell division and transfer and amino acids more with growth or enlargement of the cells. Cytokinins thus can be looked upon as catalyzing the incorporation of nucleotides into DNA and RNA, and auxins as turning on amino acid production and proteins into enzymes according to the instructions carried out of the cell nucleus by tRNA. Information transfer would precede enzyme production, and the two processes would have different effects on the organism depending upon the relative rates of the two processes. A balance between auxin and cytokinin would promote undifferentiated growth in tissue cultures, but too much cytokinin (or less auxin) could cause an accumulation of products favorable to rapid cell division and differentiation. On the other hand, less cytokinin (or more auxin) would cause cell enlargement at the expense of cell division. Rapid growth by auxin

would then be the result of enlarging cells, and not due to more cells from cell division (cytokinins).

This hypothesis can easily be tested with material already in storage and ready for preparation and observation. Tissue was placed in fixative after it was collected weekly from Test 5 material, and can be sectioned, stained and observed for large or small cells in callus collected weekly, up to four weeks after subculture. In tissue five or more weeks old, large cells with little cell division would not disprove the hypothesis. However, the preparation is tedious and requires several lengthy processes of dehydration, embedding in hot paraffin, sectioning, mounting on slides, removal of paraffin, hydration, double staining, dehydration and permanently mounting the specimen on the slide. We hope to complete this work this winter.

Dr. Dillingham, a biochemist on the Institute Staff, suggested using the popular curvature test of oat (Avena) coleoptiles (39) as the still most sensitive test for auxin. In a new study, stock tissue would be collected weekly (or some shorter interval), and blocks of tissue put directly onto prepared coleoptiles. The agar medium directly beneath the callus tissue could also be tested. The degree of curvature away from the added material would be a direct response to the amount of auxin present. By this method the endogenous production of natural auxin (IAA) in callus could be established, and whether it is a gradual increase from week to week in production, with diffusion into the medium, or if auxin appears suddenly after the tissue becomes four weeks old. The amounts of auxin necessary to show differences in the curvature test are far too small to be tested by other conventional means.

Hopefully, the observations and measurements of seasonal fluctuations in the rate of growth are also related to auxin production. Perhaps this is accomplished by turning on and off the genes for auxin production, in response to some stimulus as yet undiscovered.

In addition to immediate practical applications of tissue culture to the clonal propagation of trees, we are also at the point where spin-off information of a basic nature becomes an important link in the overall study of Why and How plants are formed, develop and mature. The mechanisms of gene action and hormonal control is now an important problem for many workers now engaged in tissue culture research, mainly because tissue culture has been shown to be such a powerful tool for working at the level of cell or tissue organization in living organisms.

NUCLEOTIDE SUPPLEMENTS

In the last section we saw that amino acid supplements were only important to tissues growing longer than four weeks before subculture. However, shoots are produced better on tissue grown for four weeks or less. During the first four weeks, the nucleotides of Group 6 (Table I) apparently increase the growth of desirable firm white tissue in stock cultures. With the emphasis on four-week old tissue for shoot production, this study was designed to determine the activity of the five nucleotides singly or in groups of four with one omitted. This tissue was then used in the shoot initiation tests described in the next section, in order to determine whether the same or different nucleotides affect tissue growth in stock cultures and shoot initiation on BAP media. So far, only that half of the study is complete that tested the nucleotides with amino acids added. Nucleotides will next be tested without amino acids.

For background, the cell chromosomes are carriers of the genes or units of inheritance. Chromosomes are made from deoxyribonucleic acid (DNA) and transfer their genetic information to the metabolic factories by different forms of ribonucleic acid (RNA). The DNA molecule is composed of two polynucleotide chains, each with a backbone of alternating pentose sugar – phosphate units, with a base attached to the sugar. Four bases are found in DNA and have either one ring and are termed pyrimidines (thymine and cytosine) or two rings and are called purines (adenine and guanine). In RNA, uracil replaces thymine. The unit composed of the base-sugar-phosphate is a nucleotide and is supplied to the nutrient medium as a monohydrate crystal. Nomenclature is guided by the base, and for adenine the nucleotide is called either adenylic acid or adenosine-3'-(2')-phosphoric acid.

In DNA, two single chains are coiled helically around each other in opposite directions, and are held together by hydrogen bonding of the specific pairing of thymine of one chain with adenine of the other, or of the guanine-cytosine bonding from opposite points on the two chains. This specific pairing and the linear order of triplicates of bases determines the genetic code discussed earlier.

Callus Growth

Tissue 1 was subcultured to two sets of media, from stock cultures initiated 1/23/67 and subcultured monthly to medium 1 for 28 passages. The basic test medium was made by adding Groups 1, 2, 3 and 4 of the supplements given in Table I to medium 1. The two sets of final test media were made by first adding one nucleotide at a time to the basic medium, then adding four at a time for the second set. Ten inocula were distributed among two dishes for each medium and placed in the dark incubator at 28°C.

At the end of the first four-week passage, all tissue appeared about the same, with a little more firm white tissue on two media containing all nucleotides except cytidylic acid and all nucleotides except adenylic acid. The best tissue of each treatment was subcultured to fresh medium and incubated for another month.

At the end of the second passage, the two best pieces per treatment were photographed (Fig. 21), and five pieces in one dish were weighed for each treatment. Initial weights were not taken, so the averages in Table VII are total wet weights rather than increments.

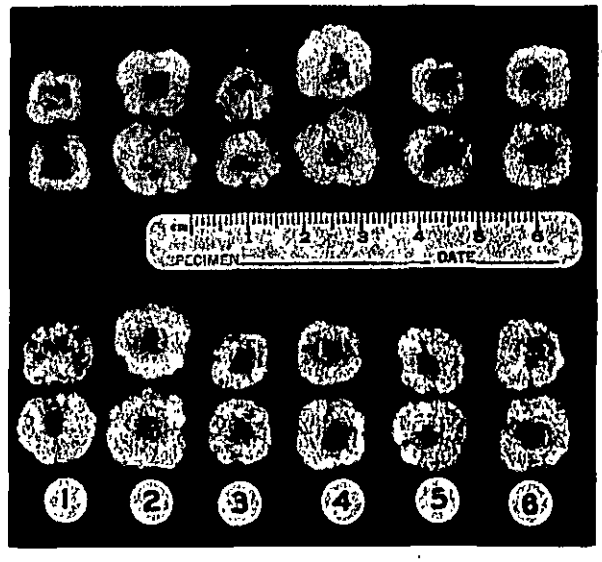

Figure 21. Tissue 1 After Four Weeks on Basic Medium with One Nucleotide at a Time Added (Top) and Four Nucleotides at a Time (Bottom). Numbers Correspond to Media Described in Table VII

TABLE VII

TOTAL WET WEIGHT OF TISSUE 1 ON NUCLEOTIDE TEST MEDIA
AFTER THE SECOND MONTHLY PASSAGE

Medium	Added to Basic Medium	Weight, mg. Av.[a]	S.D.[b]
One nucleotide at a time[c]			
4	Guanylic acid	458	± 63
2	Adenylic acid	374	± 59
1	(Medium 1)	252	± 34
6	Uridylic acid	239	± 52
3	Cytidylic acid	172	± 32
5	Thymine	171	± 49
Four nucleotides at a time[d]			
2	-CGTU	414	± 41
6	ACGT-	254	± 55
5	ACG-U	249	± 17
1	ACG TU	249	± 72
4	AC-TU	228	±100
3	A-GTU	194	± 17

[a] Averages joined by the same line are not different at the 5% level of significance by DMT (38).

[b] Standard deviation $= \sqrt{\frac{\Sigma x^2 - (\Sigma x)^2/N}{N-1}}$ calculated independently of DMT (38).

[c] Tissue on top, Fig. 21.

[d] Tissue on bottom, Fig. 21.

When added in the first set of media, adenylic acid alone caused a significant growth increase over the control tissue, and guanylic acid alone caused still a further growth increase over that obtained with adenylic acid (Table VII). The rest of the media in the first set were neither better nor worse than medium 1 according to Duncan's Multiple Test (DMT) (38).

Strangely enough, in the second set of media the only significant growth increase above the control medium 107 (and all other media) occurred in tissue on basic medium containing all of the five nucleotides except adenylic acid. Until the rest of this study is completed, perhaps it is wise to refrain from too many speculations to explain these results. However, a quick look at the theoretical pathways of DNA and RNA synthesis shows some interesting points to consider before designing the remainder of this study.

Two main pathways have been postulated for the production of nucleotides, composed of either a purine or pyrimidine base, a pentose sugar and phosphate. The pyrimidines thymidine, uridine and cytidine are formed in one pathway, while the purines guanosine and adenosine are formed in the other. For DNA, thymine and cytosine bases come from one pathway and guanosine and adenine from the other. However, when the bases bond with their opposite partner to form the double DNA helix, thymine pairs with adenine and guanine pairs with cytidine. Thus, during replication for cell division or for transcribing the genetic message for transmission outside of the nucleus, the exposed base to be paired draws its opposite partner from the metabolic pathway other than its own. In this manner, a purine always pairs with a pyrimidine, and the only two combinations found are T-A and C-G.

The two pathways may explain, in part, why the addition of guanylic acid or adenylic acid increases growth; since both are purines and come from the same pathway, indicating that precursors for purines with two rings are in limited supply, whereas the precursors for the pyrimidines thymine, uridylic acid and cytidylic acid are not limiting in our medium. Another hypothesis is that an excess of adenine inhibits growth in the presence of guanine or vice versa, because of interconversions, but obviously much more work must be done on this problem before more substantive hypotheses can be advanced and tested. Fortunately, many other workers are engaged in the same type of research, but it is an unexpected surprise that our cultures appear suitable for the study of gene regulation at the molecular level. Our linking of the availability of guanine with that of adenine may add additional insight to the two-path synthesis proposed by others, but it may also provide an opportunity to look for possible conversions from one base to another with the use of labelled units, or even look for an antagonism mechanism.

Shoot Initiation on Tissue Grown with Nucleotides

The tissue grown during the second passage of the nucleotide media test was subcultured to both medium BAP-.05 and BAP-.15 from each test medium in Table VII. After five weeks in the dark, the length and number of shoots and roots per piece were recorded and are summarized in Table VIII.

The results are difficult to interpret without the data from the rest of the uncompleted study, but adding one nucleotide at a time, both the total number of shoots and the average shoots per inoculum were about the same for all tissues (except tissue 1) on medium BAP-.15, with the best frequency with guanylic acid alone. No shoots were produced on medium BAP-.05, perhaps because it was still earlier in the season (August) than when vigorous shoots were initiated last year

on BAP-.05, in September and October. With four nucleotides added at a time, the three tissues with the highest percentages and the most total shoots all had been grown with both adenylic and guanylic acids before transfer to BAP media (treatments 8, 10 and 11, Table VIII).

TABLE VIII

SHOOT AND ROOT PRODUCTION, FIVE WEEKS AFTER THE TRANSFER OF TISSUE FROM NUCLEOTIDE TEST MEDIA TO BAP-.05 OR BAP-.15

Treatment	Source of Inocula[a] Basic Medium Plus:	Inocula, % BAP-.05 Shoots	BAP-.05 Roots	BAP-.15 Shoots	BAP-.15 Roots	No. of Shoots Total	No. of Shoots Av./Inoculum
1	-	-	70	56	11	89	1.6
2	A	-	90	60	40	140	2.3
3	C	-	90	70	40	160	2.3
4	G	-	70	80	30	150	1.9
5	T	-	100	60	70	130	2.3
6	U	-	50	70	-	140	2.0
7	- C G T U	-	30	10	20	20	2.0
8	A - G T U	-	100	90	30	230	2.6
9	A C - T U	-	60	40	10	110	2.8
10	A C G - U	-	60	70	20	210	3.0
11	A C G T -	-	50	60	40	120	2.0
12	A C G T U	-	10	10	-	10	1.0

[a] Tissue 1 grown for two four-week passages on medium 1, and media containing Groups 1, 2, 3 and 4 of Table I (Basic Medium) plus either one or four nucleotides at a time.

	Pairing			
	DNA:	A--T	RNA:	A--U
Purines: A G				
Pyrimidines: T C		G--C		G--C

Adenine plus guanine appeared to inhibit the growth of stock cultures synergistically (working together), but in this study the presence of both is a common denominator for high shoot production where four nucleotides at a time were tested. This is reminiscent of an earlier problem, that fast growth and high yield are stressed by other tissue culture workers, but for our tissue the fast-growing friable and tan callus never produced roots or shoots, but were only found on slower-growing firm and white tissue. Perhaps there is a general law to be discovered that what may be right for callus growth may not be best for differentiation into roots and shoots. We know that auxin must be removed from the medium to produce shoots. Perhaps many other changes must also occur before differentiation, such as a shift in requirements from some amino acids or nucleotides to others. This reasoning is logical from the standpoint that nucleotides and amino acids are building blocks between the genes and the enzymes, and a shift in function may necessitate a previous shift in the genetic communications to cause the shift. Different instructions mean that different codons on the gene send the new instructions by different combinations of nucleotides. Continued experiments of this type may reveal what these different requirements are, so that shoot initiation can be appreciably increased for clonal propagation, as well as increase spin-off of new knowledge of molecular botany.

Just one last thought to close this report. Shoot initiation is inhibited by auxin but stimulated by cytokinin in the absence of auxin. These last two studies have shown the importance of adenine and guanine, which appear to work synergistically to inhibit tissue growth but work synergistically to stimulate shoot initiation. Adenylic and guanylic acids are both purines: cytokinins are also purines! The question of what this means is occupying the thoughts of many people.

FUTURE PLANS

Instead of a short outline of future plans, parts of two memoranda are included that were sent to Mr. John Swanson on August 5 and September 5, 1969. The first is a proposal submitted to the Pioneering Research Committee, requesting a renewal of its support of the tissue culture program, and the second is a more detailed work schedule for the fall of 1969. The two main areas of exploration will be (1) clonal propagation of tree species from tissue cultures, and (2) cell cultures for both basic studies and eventual propagation from single cells.

PROJECT PROPOSAL

August 5, 1969

Date: July 1, 1969

Title: Vegetative Propagation of Isolated Aspen Tissue of Single Cell Origin

To: Pioneering Research Committee

BACKGROUND

For the past seven years, the main objective of this study has been to reproduce aspen trees from single cells. When we succeed, aspen breeders will have not only a rapid and inexpensive cloning method for superior trees, but foundations will have been laid for improved forest fertilization on the practical side, as well as to develop cell culture as a tool to probe molecular biology at a new level of basic research.

Single cells will not grow without a balanced medium, and a medium cannot be developed without single cells. So an indirect and intermediate approach has included the following steps:

1. Tissue culture

    a. Undefined coconut milk medium (done).
    b. Defined medium, quantitative growth data for several variables (done).
    c. Shoot initiation on callus tissue of triploid quaking aspen.

        1) Rooting on callus = complete plant, growth into tree (done).
        2) Rooting of shoots after separation from callus; growth into trees (done).
        3) Refine method for practical cloning.
        4) Repeat for other aspen species with different nutrient requirements.

2. Cell culture

    a. Cell suspensions in liquid shake cultures from tissue cultures

        1) Cells grown into colonies to form callus.
        2) Cells separate after cell division, forming an "ideal" suspension of single cells.

    b. Suspensions from leaf or branch-tip cells removed <u>in situ</u> (under study).

3. Isolation of single cells

    a. Sterile screening (done).
    b. Manually under microscope (under study).

4. Growth of single cells into embryoids, plants, then trees.

   a. Medium conditioned with nurse callus tissue (under study).
   b. Defined medium and optimum environment (under study).

5. Comparison of nutrient requirements between aspen species and possible physiological relationships.

We have reproduced triploid quaking aspen trees from tissue culture, from complete plants produced on the callus and from rooted cuttings of shoots removed from callus. These are the first trees of any kind to be reproduced from tissue culture and have elicited much interest from tree breeders around the world. Still under study are several aspects of the isolation, growth and microscopic observation of single cells. Cell culture is the next big step toward an understanding of the physiology and biochemistry of aspen.

PROPOSED PROGRAM

The study would be a continuation of the steps already outlined. A time estimate to the end of 1970 is shown below, with emphasis on cell culture but also including refinements in the tissue culture technique.

Fall-Winter 1969-70:

1. Rooted cuttings – 4-5 large experiments to obtain maximum rooting on shoots produced on callus; growth into trees.
2. Complete plants – a medium supporting higher frequencies of complete plants should also grow embryoids from single cells.
3. Manual isolation of masses of cells from leaves, separation in suspension and isolation of single cells under the microscope.
4. Growth of single cells and cell suspensions on conditioned and defined media.
5. Initiation of tissue cultures from other aspen species and special trees produced in other cooperative projects.
6. Publication of past work that includes growth studies of tissue cultures, shoot initiation and tree production, and preliminary cell suspension studies.
7. Maintenance of stock tissue cultures.

Further work is dependent upon results found this fall. Hopefully, a program can be started next spring to obtain single cells from the best source (of trees or position on a tree), and run extensive tests to determine frequencies of cell survival and embryoid development. It may be optimistic to expect a tree from a cell within the year, but it may come unexpectedly, as did the trees from tissue culture. A more realistic estimate would be 2-3 years. We are trying first to obtain growth from cells or cell colony in a suspension, then document later the growth of one cell into a tree.

The best shoot initiation occurs during the fall and winter, when competition for time from other cooperative projects is also reduced. Shoot production declines, however, during the normal growing season, when emphasis of time shifts back to stock maintenance and growth and cell studies.

EXPERIMENTAL MATERIAL

The primary experimental tissue was cultured from one tree of a natural triploid clone of quaking aspen (T-2-56) in 1967, and has been subcultured every 3-4 weeks to fresh medium. Tissues will also be cultured from other aspens, including diploid quaking aspen (Populus tremuloides Michx.), European gray poplar [P. canescens (Ait.) Sm.], European aspen (P. tremula L.), white poplar (P. alba L.), bigtooth aspen (P. grandidentata Michx.), and Eastern cottonwood (P. deltoides Marsh.).

The whole process from cell to tree will be attempted with one triploid quaking aspen tissue (T-2-56) then adapted to other aspens. An exception might be a new, recently initiated clone of triploid quaking aspen (T-38-59) that so far is outgrowing our stock T-2-56 tissue. Comparisons may be run later between the two tissues.

REPORTS

Annual report issued in October.

PROJECT DIRECTION

Principal investigator:	Dr. Lawson L. Winton Research Fellow
Assisted by:	Mrs. Shirley Verhagen Laboratory Assistant
Immediate supervisor:	Dr. Dean W. Einspahr Senior Research Associate

Division chairman: Mr. John W. Swanson
Director, Division of Natural Materials & Systems
The Institute of Paper Chemistry
Appleton, Wisconsin

FALL SCHEDULE

Triploid quaking aspen was successfully reproduced last year, from complete plants initiated on callus tissue and from shoots cut from the callus and rooted in soil or medium. Only four of seven complete plants survived from 1969, and four plants in 1968 were all lost. These were all initiated with 0.5 mg./l. of the cytokinin benzylaminopurine. The prospect is much better, however, of clonal propagation on a large scale by rooting the stunted shoots initiated on medium made with 0.15 mg./l. benzylaminopurine (BAP).

The purpose of the main experiments in tissue culture this fall will be to obtain the maximum number of shoots initiated on callus that will provide the maximum number of rooted cuttings for growth into trees.

Tissue: Triploid quaking aspen clone T-2-56, isolated January 23, 1967 on medium 1, and subcultured monthly for the past 19 months on medium 107 (medium 1 plus 28 amino acids, five nucleotides and folic acid).

Parameters: Stock tissue, grown for four weeks from subculture, will be cut to 3-mm. cubes (50-60 mg.) in October, November, December and January, and placed on medium BAP-.15 in Petri dishes wrapped in Saran, and placed in a dark incubator at 28°C. After four weeks, the number of pieces with shoots and the number of shoots per piece will be counted.

All shoots over 5-mm. tall will be cut from the callus, recorded and their bases thrust into medium 100 (medium 107 plus tryptophan) under 300 ft.-c. of balanced light at 22°C. Shoots will be kept covered with an inverted glass crystallizing dish to maintain high humidity, until leaves fail to wilt in the open. After 5-6 weeks in the light, all rooted shoots will be transplanted in soil and grown in the growth chamber. Survival will be reported as the percentage of trees grown from (1) shoots initiated and (2) rooted shoots.

OTHER TESTS

Another triploid quaking aspen clone (T-38-59) is growing vigorous firm white tissue on three media, and will be tested for a tryptophan effect in shoot initiation on media with 0.05, 0.10 or 0.15 mg./l. BAP. Other cultures (from haploid and polyploid trees produced under the 1800 Project) will similarly be tested for production of both complete plants and the rooting of isolated cuttings.

Currently, liquid cultures are now on the shaker to study embryogeny from single cells; and two replications were put out on agar medium, of tissue T-2-56 grown on media 1, 100 and 107 for four weeks, then subcultured to media with 0.05 or 0.15 mg./l. BAP.

LATE NEWS

Our fall schedule was begun, with one of the goals to improve clonal propagation of trees from rooted cuttings produced on tissue culture. In the preliminary experiment, shoots were initiated on inocula cut from stock tissue and placed on medium BAP-.05. Shoots were cut from the callus and placed in medium 100 under low light. Several cut shoots (cuttings) have rooted, and elongation or the second growth flush has started. Stock tissue was subcultured to twice the number of culture dishes last month, to increase the amount of tissue available for several large experiments this fall.

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